Effect of PSC 833, an inhibitor of P-glycoprotein on N-methyl-N-nitrosourea induced mammary carcinogenesis in rats

Janarthanan Kankesan1, Ramesh Vanama1, Aroon Yusuf1, Jake J.Thiessen2, Victor Ling3, Prema M.Rao1, Srinivasan Rajalakshmi1 and Dittakavi S.R.Sarma1,4

1Departments of Laboratory Medicine and Pathobiology and 2Pharmacy, University of Toronto, Toronto, 100 College Street, Toronto, ON MSG 1L5, Canada and 3 BC Cancer Agency and University of British Columbia, Vancouver, British Columbia, Canada

4To whom correspondence should be addressed
Email: sarma.dittakavi@utoronto.ca

Studies in our laboratory on the role of P-glycoprotein (Pgp, coded by mdr1 gene) have led to the hypothesis that over-expression of Pgp is closely associated with the development of cancer. It was conceived therefore that inhibitors of Pgp should inhibit the development of cancer. We have reported that PSC833 (PSC), a potent inhibitor of Pgp, inhibits the development of liver cancer in rats. Similarly, based on the intrinsic over-expression of Pgp in experimental mammary carcinogenesis, we studied the effect of PSC on N-methyl-N-nitrosourea induced mammary cancer in female Sprague–Dawley rats. The study indicates that PSC at daily dietary doses of 15 (PSC15) and 30 mg/kg (PSC30) body wt resulted in dose-dependent inhibition of the incidence as well as the growth of mammary tumors. Compared with controls, PSC15 and PSC30 inhibited: (i) mean tumor multiplicity by 32 and 67%, (ii) median tumor burden by 46 and 93% and (iii) incidence of ulcerated tumors by 40 and 82%, respectively. Most remarkably, PSC delayed median tumor incidence by 8 weeks, and exerted a 100% inhibitory effect on the incidence of large tumors, 4 cm3 and greater. In all the cases, although the inhibitory effect of PSC was evident at both doses, only PSC30 exhibited statistical significance. A possible compounding effect that was also observed in PSC30-treated rats was a decrease in body weight gain not attributed to diminished food consumption. All in all, consistent with recent reports, which have demonstrated inhibition of cancer development by compromising Pgp function, this study introduces a novel role for Pgp in breast cancer and potentially an unexplored therapeutic approach in treating the disease.

Introduction

Our study on the role of P-glycoprotein (Pgp, coded by mdr1 gene) in the carcinogenic process has led to the hypothesis that its over-expression is intimately associated with the development of cancer. This hypothesis is based on our findings that over-expression of Pgp begins early in experimental liver carcinogenesis and increases with the progression of the disease (1). High expression of the mdr1 gene has also been reported in many untreated human cancers such as in breast, liver, colon, kidney and brain (2–8). Taken together, these observations suggest that increased expression of Pgp in cancers plays a role in cancer development. It is probable therefore, that inhibition of Pgp function could inhibit the development of cancer. In an earlier study on liver carcinogenesis we showed that PSC833 (PSC, Novartis), a potent inhibitor of Pgp inhibited the development of liver cancer induced by 1,2-dimethylhydrazine in the rat (9). Human breast cancers often over-express Pgp (2,4,5,7,10–13). Several studies have correlated Pgp expression in breast carcinoma with poor prognosis (11,12). In human breast carcinomas, Pgp expression in neoplastic epithelial cells appears to be a marker of a more malignant phenotype especially when accompanied by stromal expression (13). Furthermore, experimental mammary tumors induced in rats by N-methyl-N-nitrosourea (MNU) also over-express Pgp (8) and the increased expression of Pgp is seen particularly at the epithelial–stroma interface. These results suggested that Pgp expression might confer an invasive advantage. Based on these considerations, the present study was undertaken to determine whether PSC, an inhibitor of Pgp would inhibit MNU-induced mammary cancer development in the rat.

Materials and methods

Reagents

PSC was a gift from Novartis. MNU (Sigma Chemical Co., St Louis, MO) was freshly dissolved in 0.9% sodium chloride adjusted to pH 6.7 with 0.05% acetic acid to give a concentration of 10 mg MNU/ml, and administered within 2 h of preparation.

Animal care

The experimental protocol used was approved by the University of Toronto Animal Care and Use Committee. Rats were housed individually in solid bottom cages with corncob bedding, at 22°C and 50% humidity (9). At day 50, all rats received a single dose of MNU (50 mg/kg i.p.). Two weeks post-MNU administration, a time by which the animals would have recovered from the MNU-induced toxicity, the rats were divided into three groups. Rats in Group 1 were exposed to basal diet AIN (n = 21), while rats in Groups 2 and 3 were exposed to AIN diet containing two different concentrations of PSC. PSC was pre-mixed at 325 and 645 mg/kg AIN diet to achieve daily PSC doses of 15 (PSC15; n = 20) and 30 mg/kg body wt (PSC30; n = 17), respectively, based on an average diet consumption of 14.0 g/day/rat. Body weight and diet consumption of the rats were monitored every week. Beginning 6 weeks post-MNU administration, the rats were palpated every week for the evidence of tumors. The position of palpable tumors was categorized as left or right cervical, thoracic, abdominal or inguinal. Dimensions (length (L) × width (W) × depth (D)) of the tumors were measured with a pair of digital calipers, and the volume was calculated using the following formula: \(0.5238 \times L \times W \times D\). Rats that developed ulcerating tumors before the termination of the experiment were killed within a day after diagnosis. At the termination of the experiment, 26 weeks post-MNU, the rats were killed by carbon dioxide asphyxiation and a detailed necropsy was performed on each rat.

Abbreviations: MNU, N-methyl-N-nitrosourea; Pgp, P-glycoprotein; PSC, PSC833.
rat. Blood was drawn from the common femoral artery at the bifurcation, and the serum was stored at −80 °C. Location, weight and dimensions of excised mammary tumors were recorded. Tumors larger than 1 cm³ in volume were sliced along the middle and fixed in 10% phosphate-buffered formalin, embedded in paraffin blocks and processed for histological examination of adenomas and adenocarcinomas using criteria described by Russo et al. (14).

Briefly, histopathologic criteria used to determine malignancy were: (i) loss of tubular-alveolar pattern of the normal mammary gland, (ii) presence of large epithelial cells with increased nucleo-cytoplasmic ratio, (iii) stromal response by fibrosis and inflammatory cell infiltration and (iv) necrosis and hemorrhage. In each case, the remaining tumor tissue was instantly frozen in liquid nitrogen and stored at −80 °C for further analysis. The kidneys, liver, lungs and surrounding mammary tissues were also removed and stored for histological and biochemical analyses.

**Western blot analysis of Pgp**

Frozen mammary tumor tissues (0.5 g) were thawed to room temperature, homogenized in 3 ml HEPES-mannitol buffer containing 50 mM mannitol, 20 mM HEPES, 10 mM β-mercaptoethanol, 1:1000 dilution of protease inhibitor cocktail (P-8340, Sigma Aldrich, St Louis, MO) and centrifuged at 15 000 g for 15 min. The supernatant was then centrifuged at 100 000 g for 60 min. The microsomal pellet was resuspended and dissolved in the HEPES-mannitol buffer. Total protein content in tissue lysates was estimated using the Bio-Rad protein assay kit with bovine serum albumin as the standard. The microsomal fractions (100 and 200 μg of protein) were electrophoresed in 8% SDS–PAGE according to standard procedures. The samples were then blotted to nitrocellulose paper and immunoprobed using C219 (1:3000, Fujirebio Diagnostics, Malvern, PA), a monoclonal antibody, which recognizes the different isoforms of Pgp. The blots were developed using the ECL Kit (Amersham Pharmaceuticals, Piscataway, NJ). Pgp expression was represented by a ~170 kDa band on the SDS–PAGE.

**Statistical analysis**

Incidence of tumors (expressed as the percentage of tumor bearing rats per group) or ulcerated tumors (expressed as the percentage of rats killed for ulcerated tumors per group) were compared among the groups using the Fisher’s exact contingency test. Cumulative tumor incidence was assessed in a time-dependent manner using Kaplan–Meier Life Table Curves. To generate these curves, rats that produced the first detectable tumor were assigned a score of 1, while those that failed to develop tumors until the end of study were given a score of 0. Three rats in the PSC30 group were killed for PSC-induced morbidity and were excluded from all analyses performed in the study. The scores were then tabulated based on the time of tumor detection, represented as weeks post-MNU administration. The curvatures of the treatment groups were then compared with those of the control group using the Log Rank test. Tumor multiplicity (expressed as number of tumors per rat) and tumor burden (expressed as total volume of tumors per rat) were compared using one-way ANOVA followed by Dunnett’s Post Comparison test.

**Results**

From our earlier studies, we found that PSC30 exhibited toxicity in rats bearing hepatic nodules but not in the initiated rats that do not have hepatic nodules (9). Since the liver appears not significantly involved in mammary cancer study, in that no nodules are seen in the liver, in the present study we elected to use PSC30 to maximize the inhibitory effect on mammary tumor development. However, dietary PSC30 resulted in toxicity characteristic of ataxia associated with decreased gain in body weight in several rats (Figure 1). Nevertheless, beginning 12 weeks post-MNU treatment, the rate of gain in body weight in PSC30 group was comparable with that in the control AIN group. Interestingly, food consumption in all the groups was similar. On average, daily diet consumption per rat in AIN, PSC15 and PSC30 groups was 16.9 ± 0.3, 17.1 ± 0.4 and 16.0 ± 0.4 g/day (mean ± SE), respectively. In addition, the ratios of liver and kidney weights to body weight (Figure 2) are comparable between groups.

As pointed out in the Introduction, several studies have reported that human breast cancers exhibit increased expression of Pgp. However, there are a few studies that did not show an increased expression of Pgp/mdr1 in human breast cancers (13,15). These discrepancies were attributed to the different methods of Pgp detection and also to variations in the definitions of positivity within a given method of detection (16,17).

A recent meta-analysis performed on 31 studies to assess functional significance of Pgp expression in breast cancers indicated that 41.2% of breast cancers express Pgp/mdr1 (18). The results of the present study indicate that the MNU-induced mammary tumors exhibited increased expression of Pgp compared with their corresponding non-lesion surrounding mammary tissue. We have screened over 15 mammary tumors in the present study and all of them exhibited increased Pgp expression. These results are in agreement with those published earlier (8). Western analysis of Pgp expression in tumors compared with surrounding from four representative rats is presented in Figure 3. Interestingly, the surrounding tissue exhibited a C219 cross-reacting band of a slightly higher molecular weight than Pgp. At present we have not characterized this protein or its relationship to the 170 kDa Pgp band found in the tumor tissue.

To determine whether PSC exerted any inhibitory effect on mammary tumor development, cumulative tumor incidence was plotted using a Kaplan–Meier Life Table Curve. The results presented in Figure 4 show that in the PSC30 group, rats had significantly decreased incidence of tumors and the inhibition was persistent until the end of the study. Further, the median tumor incidence was extended from 16 weeks in the controls to 24 weeks in PSC30. While PSC15 did not exhibit an inhibitory effect on the incidence of mammary tumors when expressed as number of tumor bearing animals (Figure 4), it clearly had an effect on tumor multiplicity (Figure 5). Compared with controls, PSC15 and PSC30 inhibited the number of tumors per rat by 31.6 and 66.7%, respectively. To determine if the treatment inhibited the growth of the tumors as well, tumor burden, expressed as total tumor volume per rat, was assessed between different groups. As presented in
Figure 6. PSC30 significantly inhibited the total volume of tumors per rat by 81.6% compared with controls, while PSC15 exhibited a marginal inhibitory effect. This marginal effect of PSC15 could be due to two rats, which showed a very high tumor burden, uncharacteristic of the rest of the rats in this group. Because of this we calculated the median, which is more representative of this group. The median tumor burden for the AIN control, PSC15 and PSC30 were 3.90, 2.11 and 0.28, respectively. Thus, the median values showed that PSC15 decreased tumor burden by 46% and PSC30 by 93%. Results presented in Figure 7 show the inhibitory effect of PSC on the distribution of tumor number categorized by their volume in the three groups. Most remarkably, PSC30 completely inhibited the incidence of large tumors (4 cm³ and greater). Furthermore, rats that developed severely ulcerated mammary tumors during the course of the study were killed within a day of diagnosis. The number of rats killed for ulcerated mammary tumors in each group is shown in Figure 8. Compared with controls where seven rats (33.3%) were prematurely killed for severely ulcerated mammary tumors, there...
were only four rats (20%) in PSC15 and one rat (5.9%) in PSC30. Consistent with these observations, microscopic analysis indicated that compared with controls where 73% of the tumors were adenocarcinomas, only 54% in the PSC15 group and 24% in the PSC30 group were adenocarcinomas (Figure 9). The tumors are ductal carcinomas with varying combinations of papillary, cribriform and comedo patterns. The site-distribution of tumors in PSC30-treated rats was similar to that of controls, thereby suggesting that PSC30-induced tumor inhibition may not be site-specific. In summary, although tumor inhibition by PSC was dose-dependent, statistically significance was achieved only in the PSC30 group.

**Discussion**

The over-expression of Pgp in cancer development appears to be both an intrinsic and an acquired phenomenon. Acquired expression of Pgp often follows exposure to drugs and is thought to play a role primarily in drug-efflux. As such, Pgp modulators currently used in experimental research as well as in clinical trials, act as adjuvant agents for other chemotherapeutic drugs (19–22). However, the intrinsic expression of Pgp in tumors appears to play a more fundamental role in promoting tumor growth and development. Therefore, tumors that intrinsically over-express Pgp during development, such as tumors in MNU-induced mammary carcinogenesis in rats, should conceivably be sensitive to Pgp inhibition. The results of the present study are in support of this consideration in that PSC, a potent inhibitor of Pgp, given at daily dietary doses of 15 and 30 mg/kg body wt inhibited the incidence as well as the
growth of MNU-induced mammary tumors in rats. However, PSC30 was associated with toxicity, manifested as ataxia and decreased gain in body weight. Interestingly, the food intake was similar in all three groups. Whether this toxicity has any role in the inhibitory effect of PSC needs to be explored. It is interesting to note that in human studies similar toxicity was reported with PSC. However, these toxic manifestations were transient and reversible upon withdrawal of PSC (23,24). Nevertheless, since the PSC15 group exhibited decreased number of tumors in the absence of toxicity or a decrease in body weight, we believe that tumor inhibition with PSC30 at least in part is independent of its associated toxicity. A titration of PSC doses between 15 and 30 mg/kg body weight would indicate the most effective and non-toxic dose. In the present study PSC was given throughout the experimental period. It should be interesting to determine the minimum period of exposure to PSC necessary to achieve maximum inhibition on tumor growth. Further, it should be equally interesting to determine whether or not the tumors resume growth upon cessation of PSC exposure.

While our work was in progress, PSC was also shown to inhibit the engraftment of KG1a/200 human leukemia cells in non-obese diabetic severe combined immunodeficient mice (25). It was also reported that Mdr1-deficient Min (ApcMin+/−Mdr1ab+/−) mice exhibited fewer intestinal polyps than ApcMin−/−Mdr1ab+/− mice (26,27). All these results indicate that Pgp, besides being a drug-efflux pump, may also play an important role in tumor development.

From the mechanistic point of view, Pgp has been implicated in several signaling pathways regulating cell differentiation, proliferation and apoptosis (28,29). Whether these pathways either singly or in combination contribute to the role of Pgp in tumor development is not clear at present. Nevertheless, it is not unreasonable to surmise that inhibition of Pgp function would inhibit tumor development. Indeed our results indicate that PSC by virtue of its inhibition of Pgp function might have induced apoptosis and/or cell cycle arrest in Pgp over-expressing mammary tumor cells while sparing the non-lesion (surrounding normal) cells. Several studies have shown that PSC induces cell cycle arrest and/or apoptosis in a variety of tumor cell lines (30–37). In some of these studies it was demonstrated that the inhibition of growth/induction of apoptosis was associated with an increase in ceramide levels. However, the mechanism of increase in the levels of ceramide appears to depend on the cell type (31,38). The elucidation of the exact pathways by which PSC affects the homeostasis of ceramide levels is currently under investigation.

The present study was driven by the hypothesis that overexpression of Pgp plays a fundamental role in promoting MNU-induced mammary carcinogenesis in rats. Therefore, PSC was used as a tool for the purpose of inhibiting Pgp function. Since tumors in this model intrinsically over-express Pgp, the effect of PSC observed in this study is tentatively believed to be primarily the effect of Pgp inhibition. However, the possibility of a PSC effect independent of Pgp expression has not been ruled out.

In summary, our studies on the inhibitory effects of PSC on mammary carcinogenesis and on liver carcinogenesis (9) together with the recently published report that PSC inhibits the engraftment of KG1a/200 human leukemia cells in severe combined immunodeficient mice (25), strongly suggest that PSC by itself may be used as a cancer chemotherapeutic agent.

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References